

-7-

ELECTRONIC AVAILABLE COPY

Brief Description of the Figures

## Figures for the First Series of Experiments

5

Figures 1A-1H

Histological analysis, immunohistochemistry, and in situ hybridization of human primary and metastatic prostatic carcinomas.

- 10 (1A-1C) Photomicrographs of primary prostatic carcinomas processed as follows: (1A) Immunohistochemical staining against p27 of a prostatic intra-epithelial neoplastic (PIN) lesion; note the intense positive immunoreactivities observed in the nuclei of the tumor cells growing into the
- 15 lumen. (1B) Immunohistochemical staining against p27 of another PIN lesion showing dysplastic changes; note the intense positive immunostaining in the nuclei of normal epithelial cell and the low-to-undetectable staining of the tumor cells dissecting the gland and growing into the
- 20 lumen. (1C) Undetectable levels of p27 protein in an invasive primary prostatic carcinoma; note the staining of a normal gland trapped into the tumor.

- (1D-1F) Photomicrographs of metastatic prostatic carcinomas processed as follows: (1D) Immunohistochemical staining
- 25 against p27 of a metastatic prostate carcinoma to lymph node; note the intense nuclear staining of both tumor cells and lymphocytes (cells in the germinal center display low p27 levels). (1E) Immunohistochemical staining against p27 of another metastatic prostate carcinoma to lymph node;

30 ~~note the intense positive immunostaining in the nuclei of~~

-8-

BEST AVAILABLE COPY

lymphocytes and the undetectable levels of p27 staining on the tumor cells. (1F) Immunohistochemical staining against p27 of a metastatic prostate carcinoma to bone; note the positive immunoreactivities in the nuclei of osteoblasts and the lack of staining of tumor cells. (1F-1G) Photomicrographs of a primary invasive prostatic carcinoma processed as follows: (1F) Low-to undetectable immunohistochemical staining against p27 in the tumor cells; note the staining of a normal gland trapped into the tumor. (1G) In situ hybridization on a consecutive section from the case illustrated in panel (1H) showing high mRNA levels of p27<sup>Kip1</sup> even in p27-negative tumor cells utilizing the anti-sense probe to p27<sup>Kip1</sup>. Original magnification (1A) through (1H) 400x.

15

#### Figures 2A-2D

In certain prostatic carcinomas p27 protein is a functional cyclin-dependent kinase inhibitor. (2A) Immunohistochemical staining correlates with the presence of p27 by immunoblotting. Tumors #1 and #2 were negative and tumor #3 positive for p27 protein expression, paralleling their IHC patterns. (2B) Immunodepletion of p27 extracts. Extracts obtained from tumors #2 and #3 were subjected to sequential depletion with antibodies specific to p27 or a non-specific rabbit anti-mouse (RaM). Following depletion, the proteins in the supernatants were resolved and the presence of p27 determined by immunoblotting. (2C) Depletion of p27 depletes heat stable cyclin-dependent kinase inhibitory activity. The supernatant shown in panel B was boiled and following clarification the soluble fraction was

BLU: AVAILABLE COPY

-9-

incubated with different amounts of recombinant cyclin E/CDK2 kinase and the degree of inhibition of cyclin E/CDK2 activity on histone H1 substrate was measured. (2D) The amount of each kinase used is shown in the panel and the bars are representative activities on an arbitrary scale. Depletion with either RaM or p27 specific antibodies did not affect the inhibitory activity of the p27 negative tumor; however, depletion of p27 from the positive tumor extract completely ablated the heat stable inhibitor activity.

### Figure 3

Recurrence-free proportion analysis of patients with primary prostate carcinoma (n=42) as assessed by time to detectable PSA. Patients who had PSA relapse were classified as failures, and patients with PSA relapse, or those who were still alive or died from other disease or lost to follow-up during the study period, were coded as censored. Time to relapse was defined as the time from date of surgery to the endpoint (relapse or censoring). Disease relapse-free survivals were evaluated using the Kaplan-Meier method and the Logrank test. A trend was observed between a p27 negative phenotype and early relapse (p=0.08).

### Figures 4A-4F

Histological analysis, immunohistochemistry, and in situ hybridization of human normal prostate and benign prostatic hyperplasia.

(4A-4C) Photomicrographs of consecutive sections of

-10-

ELECTRONIC AVAILABLE COPY

normal prostate tissue processed as follows: (4A) Immunohistochemical staining against p27; intense positive immunoreactivities are observed in the nuclei of epithelial cells in the luminal side of the acinus, with decreased reactivities in the nuclei of basal and stroma cells. (4B) In situ hybridization showing high mRNA levels of p27<sup>Kip1</sup> in both epithelial and stroma cells utilizing the anti-sense probe. (4C) In situ hybridization utilizing the sense probe to p27<sup>Kip1</sup> showing lack of signals in both epithelial and stroma cells. (4D-4F) Photomicrographs of consecutive tissue sections of a benign prostatic hyperplastic nodule processed as follows: (4D) Immunohistochemical staining against p27; note the lack or almost undetectable levels of immunoreactivity observed in the nuclei of both epithelial and stroma cells in the luminal side of the acinus, with decreased reactivities in the nuclei of basal and stroma cells. (4E) In situ hybridization showing low-to-undetectable p27<sup>Kip1</sup> transcripts also in both epithelial and stroma cells utilizing the anti-sense probe; note the strong signal of the cellular inflammatory infiltrates that serve as an internal positive control. (4F) In situ hybridization utilizing the sense probe to p27<sup>Kip1</sup> showing lack of signals in epithelial and stroma cells, as well as cellular inflammatory elements. Original magnifications: (4A), (4B) and (4C) 1000x; (4D), (4E) and (4F) 400x.

30 Figures 5A-5D

Histopathological analysis of the prostatic tissues of

BEST AVAILABLE COPY

-11-

12 month old p27+/+ (5A) and p27-/- (5B-5D) mice. Photomicrographs of tissue sections of normal prostate samples processed as follows: (5A) Hematoxylin and eosin staining of a prostate gland of a p27+/+ mouse showing well defined acini of epithelial cells surrounded by a stroma containing few fibroblasts and poor in supportive connective tissue components. (5B) Hematoxylin and eosin staining of a prostate gland of a p27-/- mouse showing multiple and complex glands and hypercellular acini of epithelial cells surrounded by fibromuscular stroma cells in a connective tissue displaying abundant supportive components. (5C and 5D) Hematoxylin and eosin stainings of a prostate gland of a p27-/- mouse, high power details, illustrating the complexity of the glands and abundant fibromuscular stroma elements (5C), as well as the hypercellularity of the acini (5D). Original magnifications: (5A) and (5B) 200x; (5C) and (5D) 400x.

20

#### Figures for the Second Series of Experiments

Figures 6A-6C Progression-free and survival curves for patients with primary prostate cancer. The Kaplan-Meier method was used to estimate overall disease free survival. The log-rank analysis was used to compare the different curves. (6A) progression was significantly reduced in patients with tumors displaying a p53-positive phenotype ( $P < 0.01$ ). (6B), progression was not related to mdm2 status. (6C), progression was significantly reduced in patients with tumors displaying a p21

-12-

LAST AVAILABLE COPY

positive phenotype ( $P=0.0165$ ).

Figures 7A-7B Diagrammatic representation of the p53-pathway (7A), and alterations that may develop during tumor progression in prostate cancer (7B). (7A) p53 regulates the expression of several genes involved in cell cycle arrest (ie, p21) and apoptosis (ie, bax). p21 binds to heterodimeric protein kinases formed by cyclins and cyclin-dependent kinases (Cdk's), blocking phosphorylation of pRB/E2F1 complexes and abrogating S-phase entry. p53 also produces an autoregulatory feed back loop by transactivating mdm2. (7B) Overexpression of mdm2 has been observed to occur in several tumor types, and it is considered an oncogenic event. Upon binding to mdm2, p53 products are transcriptionally inactivated and triggered for degradation. This will release the G1 arrest imposed, in part, by p21 and abolish the apoptotic signals of the pathway. Thus, inactivation of p53 will favor proliferative activity, immortality, and development/accumulation of further DNA damage or mutations. The increased p21 expression observed in our study could be produced via growth factor signaling, which would also impact on cyclin D1 expression. The increment of p21 does not appear to be able to control the proliferative activity of tumor cells, as attested by the association of p21 positive phenotype and high Ki67 proliferative index. Taken together, mdm2 overexpression will inactivate the p53-pathway, while increased mitogenic activity will offset the RB-pathway. The mechanistic basis for this dual requirement stems, in part, from the deactivation

-13-

[L] AVAILABLE COPY

of a p53-dependent cell suicide program that would normally be brought about as a response to unchecked cellular proliferation resulting from RB-deficiency.

5 **Figures for the Fourth Series of Experiments**

Figures 8A-8B Immunohistochemistry and in situ hybridization of human benign prostatic hyperplasia (BPH). Consecutive sections of benign hyperplastic prostate tissue were processed as follows: (8A) Immunohistochemical staining of p16 is shown. Protein expression levels are undetectable in both epithelial and stromal components. (8B) In situ hybridization shows undetectable mRNA levels of p16 in both epithelial and stromal components when the antisense probe is used.

Figures 9A-9D Immunohistochemistry and in situ hybridization of human primary prostatic carcinomas. Consecutive sections of primary human prostate cancer tissue were processed as follows: (9A) Immunohistochemical staining of p16 is shown. Lack of immunoreaction noted in the nuclei and cytoplasm of both epithelial and stromal components. (9B) In situ hybridization reveals undetectable mRNA levels of p16 in both epithelial and stromal components when the antisense probe is used. (9C-9D) Histologic analysis, immunohistochemistry, and in situ hybridization of human primary prostatic carcinoma showing p16 overexpression. Consecutive sections of primary human prostate cancer tissue were processed as follows: (9C) Immunohistochemical staining of p16 is shown. Note

BEST AVAILABLE COPY

-14-

strong brown immunoreaction observed in the nuclei of cells. Faint cytoplasmic staining is noted as well.

(9D) In situ hybridization shows high mRNA levels of p16 in epithelial cells when the antisense probe is used. A normal gland (see pointer) serves as an internal negative control in both the immunohistochemical analysis in Figure 9C and also the in situ hybridization analysis in Figure 9D.

10

Figure 10 Kaplan-Meier curves, using the log rank test, stratified by p16 groups (group A or group B) of patients with primary prostate carcinoma (n=88) as assessed by time to detectable PSA level post prostatectomy. Time to relapse was defined as the time from the date of surgery to the time of PSA elevation after surgery. The median time to relapse for group A has not been reached. The median time to relapse for group B was 46.25 months. Patients who had PSA relapse were classified as having treatment failures and tumor recurrence.

15

20



-7-

Marked-up Version to Show Changes

ELECT AVAILABLE COPY

Brief Description of the Figures5 Figures for the First Series of ExperimentsFigures 1A-1H[.]

Histological analysis, immunohistochemistry, and in situ hybridization of human primary and metastatic prostatic carcinomas.

(1A-1C) Photomicrographs of primary prostatic carcinomas processed as follows: (1A) Immunohistochemical staining against p27 of a prostatic intra-epithelial neoplastic (PIN) lesion; note the intense positive immunoreactivities observed in the nuclei of the tumor cells growing into the lumen. (1B) Immunohistochemical staining against p27 of another PIN lesion showing dysplastic changes; note the intense positive immunostaining in the nuclei of normal epithelial cell and the low-to-undetectable staining of the tumor cells dissecting the gland and growing into the lumen. (1C) undetectable levels of p27 protein in an invasive primary prostatic carcinoma; note the staining of a normal gland trapped into the tumor.

25 (1D-1F) Photomicrographs of metastatic prostatic carcinomas processed as follows: (1D) Immunohistochemical staining against p27 of a metastatic prostate carcinoma to lymph node; note the intense nuclear staining of both tumor cells and lymphocytes (cells in the germinal center display low p27 levels).

(1E) Immunohistochemical staining against p27 of another metastatic prostate carcinoma to lymph node; note the

[REDACTED] AVAILABLE COPY

-8-

intense positive immunostaining in the nuclei of lymphocytes and the undetectable levels of p27 staining on the tumor cells. (1F) Immunohistochemical staining against p27 of a metastatic prostate carcinoma to bone; 5 note the positive immunoreactivities in the nuclei of osteoblasts and the lack of staining of tumor cells. (1F-1G) Photomicrographs of a primary invasive prostatic carcinoma processed as follows: (1F) Low-to undetectable immunohistochemical staining against p27 in the tumor 10 cells; note the staining of a normal gland trapped into the tumor. (1G) In situ hybridization on a consecutive section from the case illustrated in panel (1H) showing high mRNA levels of p27<sup>Kip1</sup> even in p27-negative tumor cells utilizing the anti-sense probe to p27<sup>Kip1</sup>. Original 15 magnification (1A) through (1H) 400x.

#### Figures 2A-2D[.]

In certain prostatic carcinomas p27 protein is a functional cyclin-dependent kinase inhibitor. (2A) 20 Immunohistochemical staining correlates with the presence of p27 by immunoblotting. Tumors #1 and #2 were negative and tumor #3 positive for p27 protein expression, paralleling their IHC patterns. (2B) Immunodepletion of p27 extracts. Extracts obtained from 25 tumors #2 and #3 were subjected to sequential depletion with antibodies specific to p27 or a non-specific rabbit anti-mouse (RaM). Following depletion, the proteins in the supernatants were resolved and the presence of p27 determined by immunoblotting. (2C) Depletion of p27 30 depletes heat stable cyclin-dependent kinase inhibitory activity. The supernatant shown in panel B was boiled

[20] AVAILABLE COPY

-9-

and following clarification the soluble fraction was incubated with different amounts of recombinant cyclin E/CDK2 kinase and the degree of inhibition of cyclin E/CDK2 activity on histone H1 substrate was measured.

5 (2D) The amount of each kinase used is shown in the panel and the bars are representative activities on an arbitrary scale. Depletion with either RaM or p27 specific antibodies did not affect the inhibitory activity of the p27 negative tumor; however, depletion

10 of p27 from the positive tumor extract completely ablated the heat stable inhibitor activity.

### Figure 3[.]

15 Recurrence-free proportion analysis of patients with primary prostate carcinoma (n=42) as assessed by time to detectable PSA. Patients who had PSA relapse were classified as failures, and patients with PSA relapse, or those who were still alive or died from other disease

20 or lost to follow-up during the study period, were coded as censored. Time to relapse was defined as the time from date of surgery to the endpoint (relapse or censoring). Disease relapse-free survivals were evaluated using the Kaplan-Meier method and the Logrank

25 test. A trend was observed between a p27 negative phenotype and early relapse ( $p=0.08$ ).

### Figures 4A-4F[.]

Histological analysis, immunohistochemistry, and in situ

30 hybridization of human normal prostate and benign prostatic hyperplasia.

-10-

[REDACTED] AVAILABLE COPY

(4A-4C) Photomicrographs of consecutive sections of normal prostate tissue processed as follows: (4A) Immunohistochemical staining against p27; intense positive immunoreactivities are observed in the nuclei of epithelial cells in the luminal side of the acinus, with decreased reactivities in the nuclei of basal and stroma cells. (4B) In situ hybridization showing high mRNA levels of p27<sup>Kip1</sup> in both epithelial and stroma cells utilizing the anti-sense probe. (4C) In situ hybridization utilizing the sense probe to p27<sup>Kip1</sup> showing lack of signals in both epithelial and stroma cells.

(4D-4F) Photomicrographs of consecutive tissue sections of a benign prostatic hyperplastic nodule processed as follows: (4D) Immunohistochemical staining against p27; note the lack or almost undetectable levels of immunoreactivity observed in the nuclei of both epithelial and stroma cells in the luminal side of the acinus, with decreased reactivities in the nuclei of basal and stroma cells. (4E) In situ hybridization showing low-to-undetectable p27<sup>Kip1</sup> transcripts also in both epithelial and stroma cells utilizing the anti-sense probe; note the strong signal of the cellular inflammatory infiltrates that serve as an internal positive control. (4F) In situ hybridization utilizing the sense probe to p27<sup>Kip1</sup> showing lack of signals in epithelial and stroma cells, as well as cellular inflammatory elements. Original magnifications: (4A), (4B) and (4C) 1000x; (4D), (4E) and (4F) 400x.

30

Figure 5 A-5D[.]

BEST AVAILABLE COPY

-11-

Histopathological analysis of the prostatic tissues of 12 month old p27+/+ (5A) and p27-/- (5B-5D) mice. Photomicrographs of tissue sections of normal prostate samples processed as follows: (5A) Hematoxylin and eosin staining of a prostate gland of a p27+/+ mouse showing well defined acini of epithelial cells surrounded by a stroma containing few fibroblasts and poor in supportive connective tissue components. (5B) Hematoxylin and eosin staining of a prostate gland of a p27-/- mouse showing multiple and complex glands and hypercellular acini of epithelial cells surrounded by fibromuscular stroma cells in a connective tissue displaying abundant supportive components. (5C and 5D) Hematoxylin and eosin stainings of a prostate gland of a p27-/- mouse, high power details, illustrating the complexity of the glands and abundant fibromuscular stroma elements (5C), as well as the hypercellularity of the acini (5D). Original magnifications: (5A) and (5B) 200x; (5C) and (5D) 400x.

20

#### Figures for the Second Series of Experiments

[Figure 6. Photomicrographs of selected primary prostate carcinoma cases analyzed by immunohistochemistry utilizing mouse monoclonal antibodies PAB1801 (anti-p53, A), 2A10 (anti-mdm2, B), Ab-1 (anti-p21, C), and MIB1 (anti-Ki67, D). A, p53 nuclear overexpression in tumor cells. Note the positive nuclear staining of tumor cells at a perineural invasion site (arrow). B, mdm2 nuclear overexpression in

30

BEST AVAILABLE COPY

-12-

tumor cells. C, p21 nuclear overexpression in tumor cells. D, high Ki67 proliferative index. Note the intense Ki67 nuclear staining of tumor cells at a perineural invasion site (arrow).]

5

Figures 17.1 6A-6C Progression-free and survival curves for patients with primary prostate cancer. The Kaplan-Meier method was used to estimate overall disease free survival. The log-rank analysis was used to  
10 compare the different curves. (6A) [A], progression was significantly reduced in patients with tumors displaying a p53-positive phenotype ( $P < 0.01$ ). (6B) [B], progression was not related to mdm2 status. (6C) [C], progression was significantly reduced in patients with tumors  
15 displaying a p21 positive phenotype ( $P = 0.0165$ ).

Figures 18.1 7A-7B Diagrammatic representation of the p53-pathway (7A), and alterations that may develop during tumor progression in prostate cancer (7B). (7A)  
20 p53 regulates the expression of several genes involved in cell cycle arrest (ie, p21) and apoptosis (ie, bax). p21 binds to heterodimeric protein kinases formed by cyclins and cyclin-dependent kinases (Cdk's), blocking phosphorylation of pRB/E2F1 complexes and abrogating  
25 S-phase entry. p53 also produces an autoregulatory feed back loop by transactivating mdm2. (7B) Overexpression of mdm2 has been observed to occur in several tumor types, and it is considered an oncogenic event. Upon  
binding to mdm2, p53 products are transcriptionally  
30 inactivated and triggered for degradation. This will release the G1 arrest imposed, in part, by p21 and

BEST AVAILABLE COPY

-13-

abolish the apoptotic signals of the pathway. Thus, inactivation of p53 will favor proliferative activity, immortality, and development/accumulation of further DNA damage or mutations. The increased p21 expression  
5 observed in our study could be produced via growth factor signaling, which would also impact on cyclin D1 expression. The increment of p21 does not appear to be able to control the proliferative activity of tumor  
10 cells, as attested by the association of p21 positive phenotype and high Ki67 proliferative index. Taken together, mdm2 overexpression will inactivate the p53-pathway, while increased mitogenic activity will offset the RB-pathway. The mechanistic basis for this  
15 dual requirement stems, in part, from the deactivation of a p53-dependent cell suicide program that would normally be brought about as a response to unchecked cellular proliferation resulting from RB-deficiency.

[Figures for the Third Series of Experiments

20

Figure 9A., Figure 9B., Figure 9C., Figure 9D., Figure 9E., and Figure 9F.]

Figures for the Fourth Series of Experiments

25

Figures [10.] 8A-8B Immunohistochemistry and in situ hybridization of human benign prostatic hyperplasia (BPH). Consecutive sections of benign hyperplastic  
prostate tissue were processed as follows: (8A)

30 Immunohistochemical staining of p16 is shown. Protein expression levels are undetectable in both epithelial

-14- [UNAVAILABLE COPY]

and stromal components. (8B) In situ hybridization shows undetectable mRNA levels of p16 in both epithelial and stromal components when the antisense probe is used.

5 Figures [11.] 9A-9D Immunohistochemistry and in situ hybridization of human primary prostatic carcinomas. Consecutive sections of primary human prostate cancer tissue were processed as follows: (9A) Immunohistochemical staining of p16 is shown. Lack of  
10 immunoreaction noted in the nuclei and cytoplasm of both epithelial and stromal components. (9B) In situ hybridization reveals undetectable mRNA levels of p16 in both epithelial and stromal components when the antisense probe is used. (9C-9D) Histologic analysis,  
15 immunohistochemistry, and in situ hybridization of human primary prostatic carcinoma showing p16 overexpression. Consecutive sections of primary human prostate cancer tissue were processed as follows: (9C) Immunohistochemical staining of p16 is shown. Note  
20 strong brown immunoreaction observed in the nuclei of cells. Faint cytoplasmic staining is noted as well. (9D) In situ hybridization shows high mRNA levels of p16 in epithelial cells when the antisense probe is used. A normal gland (see pointer) serves as an internal  
25 negative control in both the immunohistochemical analysis in Figure 9C and also the in situ hybridization analysis in Figure 9D.

30 Figure [12.] 10 Kaplan-Meier curves, using the log rank test, stratified by p16 groups (group A or group



BEST AVAILABLE COPY

-15-

B) of patients with primary prostate carcinoma (n=88) as assessed by time to detectable PSA level post prostatectomy. Time to relapse was defined as the time from the date of surgery to the time of PSA elevation 5 after surgery. The median time to relapse for group A has not been reached. The median time to relapse for group B was 46.25 months. Patients who had PSA relapse were classified as having treatment failures and tumor recurrence.

10